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Biodegradation of mixtures of chlorophenoxyalkanoic acid herbicides by *Alcaligenes denitrificans*

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An *Alcaligenes denitrificans* strain able to degrade (*R*)-2-(2-methyl-4-chlorophenoxy)propionic acid [(*R*)-MCPP, mecoprop] was assessed for its ability to utilise a range of chlorophenoxyalkanoic acid herbicides in single, binary, tertiary and quaternary combinations in batch culture. Degradation rates were rapid with single growth substrates; complete degradation occurred within 29 h for 2,4-dichlorophenoxyacetic acid (2,4-D), 43 h for 4-chloro-2-methylphenoxyacetic acid (MCPA) and 50 h for (*R*)-MCPP, respectively. After 20 h, the degradation of (*RS*)-2-(2,4-dichlorophenoxy)propionic acid [(*RS*)-2,4-DP] had ceased, with only the (*R*)-enantiomer being degraded. In binary combination, 2,4-D and MCPP degraded within 55 h. Degradation rates decreased when herbicides were added in tertiary and quaternary combinations. Thus, at the whole cell level, catalysis of closely related herbicides is likely to be facilitated by diverse enzymatic activity in *A. denitrificans*. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 255–259.

Keywords: *Alcaligenes denitrificans*; biodegradation; mecoprop; 2,4-D; dichlorprop

Introduction

The phenoxyalkanoic acid herbicides are xenobiotic analogues of the endogenous plant growth regulator, indole acetic acid (IAA). They are widely used in agriculture as they increase crop yields [5]. It is important to investigate the microbial degradation of this group of herbicide [7]. This progressive increase in application has resulted in many soil and aquifer microflora capable of degrading a wide variety of chlorinated aromatic compounds [2,9,27,30] including the phenoxyalkanoic acid herbicides [15,16,18,31,35].

The degradation of individual phenoxy herbicides is well documented, especially for 2,4-dichlorophenoxyacetic acid (2,4-D) [3,8,13,21]. This has been facilitated by the diversity of organisms able to utilise 2,4-D as the sole carbon source thus providing axenic cultures to elucidate the involvement of the *tfd* genes in the assemblage of the degradative pathway [6,33,34]. Similar studies on (*R*)-2-(2-methyl-4-chlorophenoxy)propionic acid (MCPP) degradation have been hampered by the limited number of equivalent pure cultures. More recently bacterial strains have been isolated with the ability to degrade MCPP (Refs. [18,31,35]; Smejkal *et al.*, unpublished) and the MCPP degradation pathway has been elucidated [32,35].

Many phenoxyalkanoic herbicides are applied to cereal crops as mixed formulations to increase the spectrum of weeds killed. Similar mixtures of these herbicides are also found in herbicide manufacturing plant effluents. Therefore, understanding the dynamics of mixed herbicide biodegradation is of great importance. Simultaneous degradation of 2,4-D and mecoprop occurs using mixed bacterial cultures derived from soil [10,22,23]. Reports have suggested that bacterial strains degrading the phenoxyacetic acid herbicides 2,4-D and 4-chloro-2-methylphenoxyacetic acid (MCPA) are unable to degrade the phenoxypropionic acids MCPP and 2-(2,4-dichlorophenoxy)propionic acid (2,4-DP) [11,24]. Some strains were even constrained to the degradation of 2,4-D as a sole carbon source [14]. The selection of mecoprop degrading strains is extremely useful as they have the ability to utilise and degrade both the phenoxypropionic and phenoxyacetic herbicides [4,12,18,31,35]. Although utilisation of alternative phenoxy herbicides by prominent strains has been investigated [11,24], there are limited studies on the biodegradation of combinations of two or more phenoxyalkanoic herbicides [29,30]. This study investigates the ability of *Alcaligenes denitrificans* to utilise a broad spectrum of the phenoxyalkanoic acid herbicides in single, binary, tertiary and quaternary mixtures. This is of extreme importance when assessing the capability of soil bacteria to facilitate degradation of mixtures of these herbicides and can also be a measure of relative persistence in soils.

Materials and methods

Chemicals

Technical grade (*R*)-MCPP and MCPA were purchased from Lancaster Synthesis (Morecambe, Lancashire, UK). MSS Optica, the potassium salt of (*R*)-MCPP, was donated by Pennine Chemical Services Ltd. (Huddersfield, West Yorkshire, UK). Technical grade 2,4-D and (*RS*)-2,4-DP were obtained from Sigma Chemicals (Poole, Dorset, UK). Analytical grade (*R*)-MCPP, 2,4-D, MCPA and (*RS*)-2,4-DP were obtained from Promochem Ltd. (St. Albans, Hertfordshire, UK).

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Herbicide stock solutions

(*R*)-MCPP, MCPA and (*RS*)-2,4-DP were made as stock solutions of 100 g l^{-1}. For this 10.0 g herbicide was added to 100 ml of 0.5 M sodium hydroxide (NaOH) solution and stirred until dissolved, the pH was then adjusted to 7.0. To prevent thermal dechlorination that may occur during conventional autoclaving, the solutions were filter sterilised, using a 0.2-μm pore size Sartorious minisart filter. The 2,4-D stock solution was prepared in the same manner but using 1.0 g of herbicide to give a concentration of 10.0 g l^{-1}.

Growth conditions

The axenic culture of *A. denitrificans* was maintained in 100 ml of minimal medium containing (g l^{-1} of distilled water): $MgSO_4$, 0.2; $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 0.5; K_2HPO_4, 1.5. To this growth medium 1.0 ml of trace element (TES) was added, which contained (g l^{-1} distilled water): Na_2EDTA, 12.0; $FeSO_4$, 2.0; $CaCl_2$, 1.0; $NaSO_4$, 10.0; $ZnSO_4$, 0.4; $MnSO_4$, 0.4; $CuSO_4$, 0.1; Na_2MoO_4, 0.1; and 0.5 ml of concentrated H_2SO_4. The pH of the solution was adjusted to 7.0. The appropriate herbicide was added to give a concentration of 1.0 g l^{-1}.

Herbicide biodegradation

For biodegradation experiments four flasks, each containing 100 ml of minimal medium, 1.0 ml of TES and 1.0 g l^{-1} of the appropriate herbicides were pre-warmed at 28°C for 1–2 h. A 2.0% inoculum of a 24-h culture of *A. denitrificans* was added to three of the flasks, the fourth flask remained uninoculated as a control. *A. denitrificans* cells used in all of the experiments had prior exposure for 24 h to a growth medium containing a combination of 1.0 g l^{-1} of (*R*)-MCPP and 2,4-D. The culture was aerated by shaking it in an orbital shaker at 120 rpm. The temperature was kept at 28°C, the optimum temperature for growth of *A. denitrificans*. Growth of the cells was measured by culture absorbance at 420 nm using a Cecil spectrophotometer. Herbicide degradation was monitored indirectly by chloride ion release and directly by HPLC. Herbicide combinations used were: (a) binary: (*R*)-MCPP and 2,4-D, (b) tertiary: (*R*)-MCPP, 2,4-D and MCPA, (c) quaternary: (*R*)-MCPP, 2,4-D, MCPA and (*RS*)-2,4-DP.

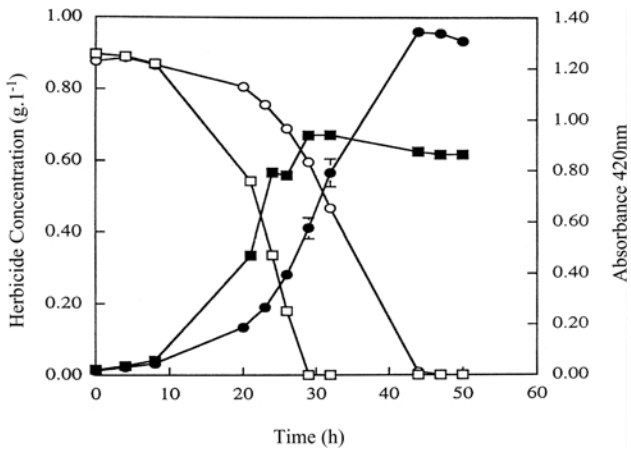

Figure 1 Decrease in concentration of 2,4-D (□) and MCPA (○) in monosubstrate cultures and the concomitant increase in absorbance of 2,4-D (■) and MCPA (●) cultures. Bars = SE, $n = 3$.

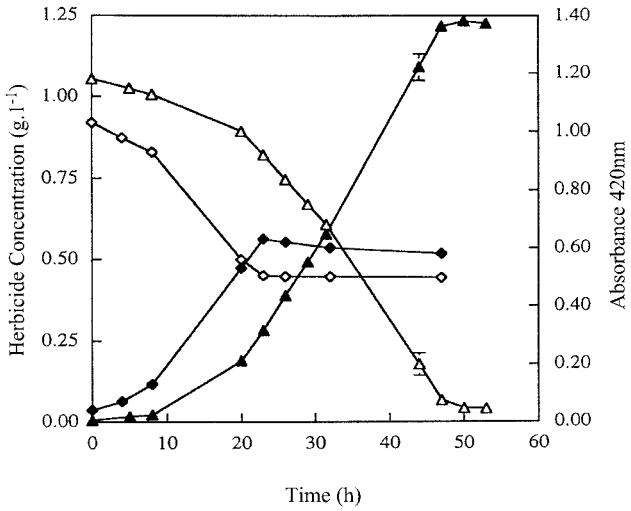

Figure 2 Decrease in concentration of (*R*)-MCPP (△) and (*RS*)-2,4-DP (◇) in monosubstrate cultures and the concomitant increase in absorbance of (*R*)-MCPP (▲) and (*RS*)-2,4-DP (◆) cultures. Bars = SE, $n = 3$.

HPLC analysis

The HPLC system consisted of a pump (Altex), an injector with a 20-μl loop (Rheodyne 7125), and LC-UV detector (Pye Unicam), and an integrator (Shimadzu C-R6A Chromatopac). A 5.0 μm Phenomenex ODS 1 column was eluted with a mobile phase of acetonitrile and phosphate buffer (50:50 v/v) at a flow rate of 1.0 ml min^{-1}, the phosphate buffer of 6.0 g K_2HPO_4 and 3.0 ml of concentrated orthophosphoric acid per litre of HPLC-grade water. The detection signal for all herbicides was monitored at 229 nm. For standard stock solutions of (*R*)-MCPP, 2,4-D, MCPA, and (*RS*)-2,4-DP the compounds were dissolved in 10.0 ml of 0.5 M NaOH before making up the volume to 100 ml with HPLC-grade water. Samples (1.5 ml) of culture were removed from each flask and transferred to an Eppendorf tube, which was centrifuged at 13,000 rpm for 10 min using a Micro Centaur centrifuge; 1.0 ml of the supernatant phase was filter sterilised, using a 0.2-μm Sartorius ministart, into a 5.0-ml vial. The samples were diluted with 1.0 ml 0.5 M NaOH, 1.0 ml HPLC-grade water and 2.0 ml 1.0 M acetic acid. To identify peaks, the retention times recorded with the experimental samples were compared with those of known reference samples. Standard curves were plotted using peak area versus concentrations of standards. HPLC data was used to determine biodegradation rates (milligrams per hour, mg h^{-1}) and percentage biodegradation (%BD) of single and mixed herbicides. Recovery of the parent compounds was estimated to be 60%.

Statistical analysis

All experiments were undertaken in triplicate and the means and standard errors calculated. When appropriate data were compared using ANOVA and considered significant if $P<0.05$ and a comparison between means was performed using a Tukey test.

Table 1 Time taken for the biodegradation of each phenoxy acid herbicide in both single- and mixed-formulation batch cultures

Herbicide	Herbicide combination							
	Single		Binary		Tertiary		Quaternary	
	%BD	Hours	%BD	Hours	%BD	Hours	%BD	Hours
(*R*)-(+)-MCPP	97	50	97	50	97	55	90	70
2,4-D	100	29	100	50	99	55	90	70
MCPA	100	43	ND	ND	60	75	40	95
(±)-2,4-DP	50	20	ND	ND	ND	ND	50	70

%BD = percentage biodegradation, ND = not determined.
Single = one herbicide present; binary = (*R*)-MCPP and 2,4-D; tertiary = (*R*)-MCPP, 2,4-D and MCPA; quaternary = (*R*)-MCPP, 2,4-D, MCPA and 2,4-DP.

Correlations were calculated using the Pearson linear correlation coefficient. All statistical calculations were carried out using the Student SYSTAT 1.0 computer package.

Results

Single-substrate biodegradation

A. denitrificans was tested for the ability to degrade 2,4-D, MCPA, (*R*)-MCPP, and (*RS*)-2,4-DP as single substrates (Figures 1 and 2). The degradation of 2,4-D was rapid and complete degradation was reached after 29 h (Figure 1, Table 1). MCPA was also rapidly degraded with complete mineralization after 43 h (Figure 1, Table 1). The overall degradation rates for 2,4-D and MCPA were 33 and 28 mg h^{-1}, respectively (Table 2). After 50 h, 97% of (*R*)-MCPP was degraded (Figure 2, Table 1) at a rate of 22 mg h^{-1}. (*RS*)-2,4-DP was degraded at a significantly faster rate (26 mg h^{-1}) than (*R*)-MCPP (Table 2); however, at 20 h only 50% of the racemic compound had been degraded (Figure 2, Table 1).

Binary-substrate biodegradation

A. denitrificans was tested for the ability to degrade a binary mixture of (*R*)-MCPP and 2,4-D with 97% and 100% mineralization at 55 h (Figure 3, Table 1), respectively. The degradation rate for (*R*)-MCPP was not significantly affected by the presence of 2,4-D; however, the rate of 2,4-D degradation was significantly slower (Table 2).

Table 2 Biodegradation rates of four phenoxy herbicides as single- and mixed-formulation batch cultures

Herbicide	Herbicide combination and biodegradation rate (mg h^{-1})			
	Single	Binary	Tertiary	Quaternary
(*R*)-(+)-MCPP	22	20	18	13
2,4-D	33	18	15	15
MCPA	28	ND	13	5
(±)-2,4-DP	26	ND	ND	6

ND = not determined.
Single = one herbicide present; binary = (*R*)-MCPP and 2,4-D; tertiary = (*R*)-MCPP, 2,4-D and MCPA; quaternary = (*R*)-MCPP, 2,4-D, MCPA and 2,4-DP.

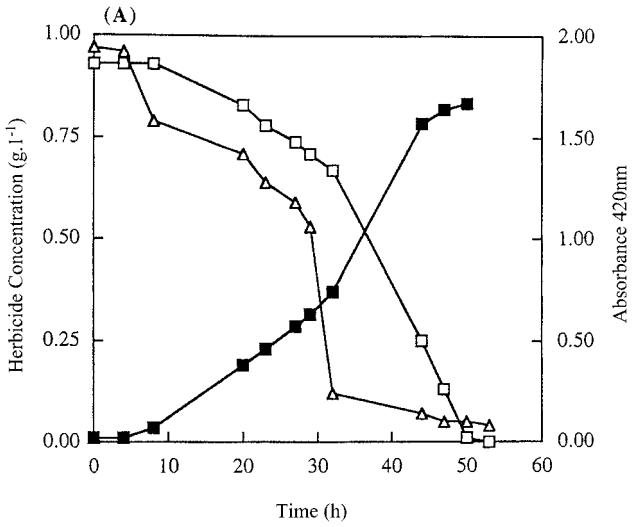

Figure 3 Binary substrate culture biodegradation of (*R*)-MCPP (△) and 2,4-D (□) with the associated increase in absorbance (■). Bars = SE, $n = 3$.

Tertiary-substrate biodegradation

Degradation of the tertiary mixture containing (*R*)-MCPP, 2,4-D and MCPA was assessed (Figure 4). The degradation patterns observed for (*R*)-MCPP and 2,4-D were approximately the same as when incorporated into a binary mixture (Tables 1 and 2). Conversely, MCPA degradation was affected by the presence of both 2,4-D and (*R*)-MCPP as only 60% was degraded as opposed to 100% when utilised as a single substrate. The rate was also significantly reduced from 28 to 13 mg h^{-1} (Table 2).

Quaternary-substrate biodegradation

The degradation of the quaternary mixture of (*R*)-MCPP, 2,4-D, MCPA and (*RS*)-2,4-DP by *A. denitrificans* was also assessed

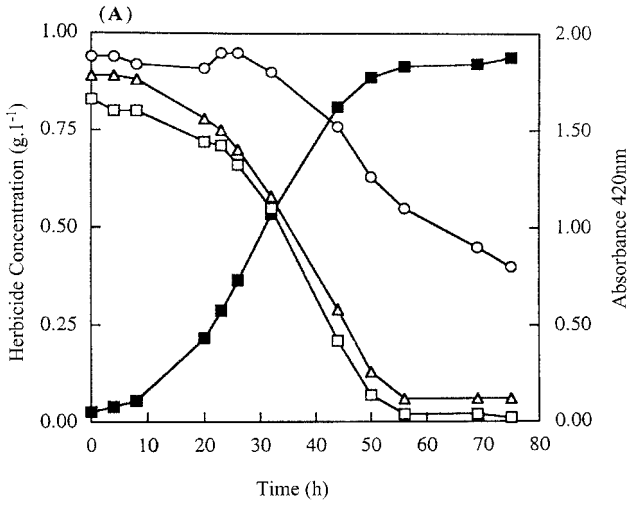

Figure 4 Biodegradation of a tertiary herbicide mixture containing (*R*)-MCPP (△), 2,4-D (□), and MCPA (○) showing associated increase in growth as absorbance at 420 nm (■). Bars = SE, $n = 3$.

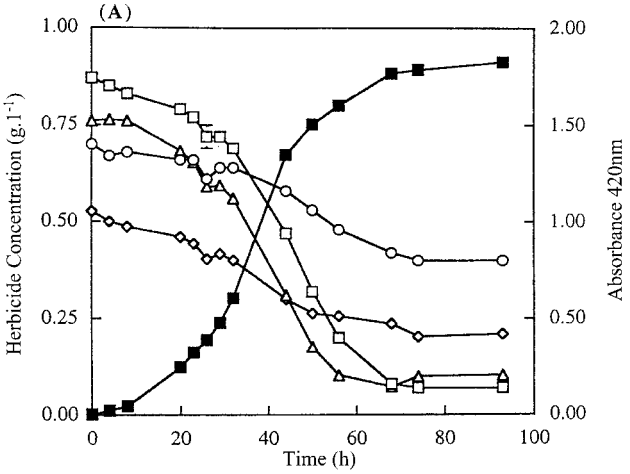

Figure 5 Biodegradation of a quaternary herbicide mixture containing (*R*)-MCPP (△), 2,4-D (□), MCPA (○), and (RS)-2,4-DP (◇) with the associated increase in absorbance (■). Bars = SE, $n = 3$.

(Figure 5). The degradation rate of (*R*)-MCPP and 2,4-D remained relatively unchanged (Table 2) with 90% biodegradation of these compounds being achieved at maximum growth (70 h). The rate of MCPA was significantly reduced to 5 mg h^{-1} with only 40% of the compound being degraded. As with single-substrate degradation, only 50% (*RS*)-2,4-DP was utilised in a quaternary mixture; however, the rate was reduced from 26 to 6 mg h^{-1} (Table 2).

Discussion

In this study we report the ability of *A. denitrificans* to degrade chlorophenoxyalkanoic acid herbicides in single and mixed combinations. It has been reported that this strain has specificity for the (*R*)-enantiomer of MCPP and is unable to degrade the (*S*)-enantiomer [31]. Further work has demonstrated that this strain also has enantiomeric specificity for the (*R*)-enantiomer of 2,4-DP [26] and is supported by the results of this study where only 50% of the racemic mixture was degraded. Many reports have encompassed the study of enantioselective uptake and degradation of chiral compounds [17,28,31,35,36] and it is believed that this is controlled by activation of enantiomer-specific enzymes [20,26]. Such discrimination between the (*R*) and (*S*)-enantiomers is often determined by the initial step of the catabolic pathway [28] where enantiomer- and substrate-specific degradative enzymes are involved. An (*RS*)-MCPP-degrading *Sphingomonas herbicidovorans* MH, was found to carry two distinct α-ketoglutarate dependent dioxygenases, one being specific for the (*R*)-enantiomer and the other for the (*S*), providing this strain with the ability to utilise both MCPP enantiomers [20].

The rate of (*RS*)-2,4-DP degradation was greater than (*R*)-MCPP despite the culture being enriched on the latter. This may be explained by the additional methyl substituent found on the aromatic ring [1]. Single substrate degradation of both 2,4-D and MCPA was achieved after 1–2 days and this is in accordance with data presented for other MCPP-degrading strains [18]. These compounds are achiral and possess a two-, rather than a three-carbon carboxylic acid side chain, thus increasing their susceptibility to degradation.

A. denitrificans effectively degraded 2,4-D and (*R*)-MCPP in binary, tertiary and quaternary combinations and this can be explained by similarities in the *tfdA* gene that encodes α-ketoglutarate-dependent dioxygenase in both MCPP- and 2,4-D-degrading strains (Ref. [26]; Smejkal *et al.*, unpublished). Additionally, in this study *A. denitrificans* had prior exposure to these compounds leading to activation of enzymes with the ability to utilise both 2,4-D and (*R*)-MCPP. The degradation of MCPA was significantly affected in tertiary and quaternary combinations and it is difficult to postulate a feasible explanation for this. Changes in pH has been shown to effect degradation [19,25] by reducing available phosphates.

A. denitrificans has the ability to degrade a wide range of phenoxy herbicides in single and mixed combinations. In biotechnological terms this is extremely useful because effluents containing mixtures of these herbicides can be treated.

The molecular profile of *A. denitrificans* is currently being investigated in our laboratory to determine the role of the *tfd* genes in the assemblage of the mecoprop metabolic pathway. Further studies will determine the levels of enzyme expression when *A. denitrificans* is exposed to varying substrates, providing valuable information on substrate preference in this strain.

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